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(54) Title: AN ENZYME EXHIBITING PECTIN METHYLESTERASE ACTIVITY (57) Abstract <p>An enzyme exhibiting pectin methylesterase activity which enzyme a) is immunologically reactive with an antibody raised against a purified pectin methylesterase derived from <i>Aspergillus aculeatus</i>, CBS 101.43, and/or b) is encoded by the DNA sequence shown in SEQ No 1 or an analogue of said sequence, and/or c) has the amino acid sequence shown in SEQ ID No. 2 or a sequence which is at least 80 % homologous thereto. The enzyme may be prepared by recombinant DNA techniques and may be used for demethylation of pectin.</p>		

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AN ENZYME EXHIBITING PECTIN METHYLESTERASE ACTIVITY

FIELD OF INVENTION

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The present invention relates to an enzyme with pectin methylesterase (PME) activity, a DNA construct encoding the enzyme, a method of producing the enzyme, an enzyme preparation containing the enzyme and various uses of the enzyme.

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BACKGROUND OF THE INVENTION

Pectin polymers are important constituents of plant primary
15 cell walls. They are mainly composed of chains of 1,4-linked α -D-galacturonic acid and methylated as well as acetylated derivatives thereof. The use of pectin-degrading enzymes is important in the food industry, primarily for fruit and vegetable processing such as fruit juice production or wine
20 making, where their ability to catalyse the degradation of the backbone of the pectin polymer is utilised.

An assortment of different pectin degrading enzymes is known to be present in various microorganisms such as *Aspergillus niger*.
25 Of these, pectin methylesterase catalyses the removal of methanol from pectin, resulting in the formation of pectic acid (polygalacturonic acid). Pectate lyase cleaves glycosidic bonds in polygalacturonic acid by β -elimination, pectin lyase cleaves the glycosidic bonds of highly methylated pectins by β -
30 elimination, and polygalacturonase hydrolyses the glycosidic linkages in the polygalacturonic acid chain.

The nucleotide and derived amino acid sequence of an *A. niger* pectin esterase cDNA sequence is disclosed by Khanh et al.
35 (1990). EP 388 593 discloses the recombinant production of an *A. niger* pectin esterase in *A. awamori* or *A. niger*. Khanh et al. (1992) disclose the effect, on enzyme yield, of using various promoters in the expression of an *A. niger* pectin methyl esterase in *A. niger*.

Markovic and Jörnvall (1992) have analyzed disulfide bridges in a tomato pectin esterase and suggest the number and location of disulphide bridges in other known and distantly related pectin esterases from *A. niger*, *Erwinia chrysanthemi* and *Pseudomonas solanacearum*. Furthermore, various amino acid residues conserved between the various enzymes are identified and a possible location of the active site of the enzymes is suggested.

10 van Rijssel et al. (1993) disclose a protein complex isolated from *Clostridium thermosaccharolyticum* which has pectin methylesterase activity. WO 93/13212 discloses a tomato pectin esterase cDNA sequence.

15 WO 93/09683 discloses the use of a purified *A. niger* pectin esterase in the production of juice from fruits and vegetables.

SUMMARY OF THE INVENTION

20 It is an object of the present invention to prepare a single-component pectin methylesterase (PME).

Accordingly, the present invention relates to an enzyme exhibiting PME activity, which enzyme

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a) is immunologically reactive with an antibody raised against a purified PME derived from *Aspergillus aculeatus*, CBS 101.43, and/or

30 b) is encoded by the coding part of the DNA sequence shown in SEQ ID No. 1 or a sequence homologous thereto encoding an enzyme exhibiting PME activity, and/or

c) has the amino acid sequence shown in SEQ ID No. 2 or is at
35 least 80% homologous with said sequence.

In the present context, the term "derived from" as used in connection with property a) is intended not only to indicate a PME produced by strain CBS 101.43, but also a PME encoded by a

DNA sequence isolated from strain CBS 101.43 and produced in a host organism transformed with said DNA sequence.

In a further aspect, the invention relates to an enzyme
5 exhibiting PME activity, which enzyme is encoded by a DNA
sequence comprising the following partial sequence

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TAAAGAGTCG   ATCATACACT   CATATCAATC   GCAAAAATGG   TTAAATCAGT
CTTGGCTTCC   GCTCTCTTCG   CCGCGTCCGC   ACTGGCTGCC   AGCCGTACCA
10 CGGCTCCCTC   CGGCGCGATC   GTCGTCGCCA   AGTCTGGTGG   TGACTATACC
ACTATTGGTG   ATGCCATTGA   TGCTCTGAGC   ACCAGCACCA   CCGACACCCA
AACCATTTTC ATCGAGGAG GGTACCTAC GATGAGCAGG TCTACCTGCC TGCTATGACC
GGCAAGGTCA TCATCTACGT CAAACCGAGA ACACCGACTC CTACG
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15 or a sequence homologous thereto encoding a polypeptide with
PME activity.

In the present context, the term "homologous" is intended to indicate a DNA which hybridizes to the same probe as the DNA
20 coding for the PME enzyme under certain specified conditions (such as presoaking in 5xSSC and prehybridizing for 1 h at -40°C in a solution of 5xSSC, 5xDenhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 µg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution
25 supplemented with 50 µCi 32-P-dCTP labelled probe for 18 h at -40°C followed by washing three times in 2xSSC, 0.2% SDS at 40°C for 30 minutes). More specifically, the term is intended to refer to a DNA sequence which is at least 80% homologous to the sequence shown in SEQ ID No. 1, such as at least 85% and
30 preferably at least 90% or 95% homologous to this sequence. The term is intended to include modifications of the DNA sequences, such as nucleotide substitutions which do not give rise to another amino acid sequence of the PME but which correspond to the codon usage of the host organism into which
35 the DNA construct is introduced or nucleotide substitutions which do give rise to a different amino acid sequence and therefore, possibly, a different protein structure which might give rise to a PME mutant with different properties than the native enzyme. Other examples of possible modifications are

insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence, or deletion of one or more nucleotides at either end or within the sequence.

5

The term "homologous" as used in connection with a polypeptide or protein is intended to indicate the degree of identity with the amino acid sequence shown in SEQ ID No. 2, which may be determined by methods known in the art. Furthermore, the
10 homologous polypeptide is preferably one which is encoded by an analogue (as defined above) of the DNA sequence shown in SEQ ID No. 1.

Furthermore, the invention relates to an enzyme exhibiting PME
15 activity, which enzyme is encoded by a DNA sequence comprising at least one of the following partial sequences

- (a) TAAAGAGTCG ATCATACACT
- (b) CATATCAATC GCAAAAATGG
- 20 (c) TTAAATCAGT CTTGGCTTCC
- (d) GCTCTCTTCG CCGCGTCCGC
- (e) ACTGGCTGCC AGCCGTACCA
- (f) CGGCTCCCTC CGGCGCGATC
- (g) GTCGTGCGCA AGTCTGGTGG
- 25 (h) TGACTATACC ACTATTGGTG
- (i) ATGCCATTGA TGCTCTGAGC
- (j) ACCAGCACCA CCGACACCCA
- (k) AACCATTTTC ATCGAGGAG
- (l) GGTACCTAC GATGAGCAGG
- 30 (m) TCTACCTGCC TGCTATGACC
- (n) GGCAAGGTCA TCATCTACGT
- (o) CAAACCGAGA ACACCGACTC CTACG

In a further aspect the invention relates to a DNA construct
35 comprising a DNA sequence encoding an enzyme exhibiting pectin methyl esterase activity, which DNA sequence comprises the coding part of the DNA sequence shown in SEQ ID No. 1 or any of the partial DNA sequence shown above, or is an analogue of said sequence, which

i) hybridizes with an oligonucleotide probe prepared on the basis of any of the partial DNA sequences shown above, of the DNA sequence shown in SEQ ID No. 1, or of the amino acid sequence shown in SEQ ID No. 2,

5

ii) encodes an enzyme comprising an amino acid sequence being at least 80% homologous with the amino acid sequence shown in SEQ ID No. 2 such as at least 85%, 90% or 95% homologous with said sequence, and/or

10

iii) encodes an enzyme which is immunologically cross-reactive with the polypeptide comprising the amino acid sequence shown in SEQ ID No. 2.

15

DETAILED DESCRIPTION OF THE INVENTION

The enzyme of the invention may be isolated by a general method involving

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- cloning, in suitable vectors, a DNA library from *Aspergillus aculeatus*,
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to
- 25 express any enzyme of interest encoded by a clone in the DNA library, and
- screening for positive clones by determining any PME activity of the enzyme produced by such clones.

30 A more detailed description of this screening method is given in Example 1 below.

The DNA sequence coding for the enzyme may for instance be isolated by screening a cDNA library of *Aspergillus aculeatus*,
35 e.g. strain CBS 101.43, publicly available from the Centraalbureau voor Schimmelcultures, Delft, NL, and selecting for clones expressing the appropriate enzyme activity (i.e. PME activity as defined by the ability of the enzyme to hydrolyse methylester bonds in pectin). The appropriate DNA sequence may

then be isolated from the clone by standard procedures, e.g. as described in Example 1. It is expected that DNA encoding a homologous enzyme may be isolated by similarly screening a cDNA library of another microorganism, in particular a fungus, such as a strain of an *Aspergillus* sp., in particular a strain of *A. aculeatus*, *A. oryzae* or *A. niger*, a strain of a *Trichoderma* sp., in particular a strain of *T. harzianum* or *T. reesie*, a strain of a *Fusarium* sp., in particular a strain of *F. oxysporum*, a strain of a *Humicola* sp. or a strain of a *Geotricum* sp.

Alternatively, the DNA coding for a PME of the invention may, in accordance with well-known procedures, conveniently be isolated from DNA from any of the above mentioned organisms by use of synthetic oligonucleotide probes, prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of any of the partial nucleotide sequences (a)-(o) listed above.

The DNA sequence may subsequently be inserted into a recombinant expression vector. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the PME should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the PME, the promoter and the

terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY, 1989).

5

The host cell which is transformed with the DNA sequence encoding the enzyme of the invention is preferably a eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a
10 species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus oryzae* as a host
15 microorganism is described in EP 238 023 (of Novo Industri A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, e.g. a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*.

20 In a still further aspect, the present invention relates to a method of producing an enzyme according to the invention, wherein a suitable host cell transformed with a DNA sequence encoding the enzyme is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered
25 from the culture.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed PME may conveniently be secreted into
30 the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion
35 exchange chromatography, affinity chromatography, or the like.

The thus purified PME may be employed for immunization of animals for the production of antibodies. More specifically, antiserum against the PME of the invention may be raised by

immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immuno-electrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, 5 Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ((NH₄)₂ SO₄), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical 10 characterization of proteins may be done either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immuno-electrophoresis (N. Axelsen et al., supra, Chapters 3 and 15 4), or by rocket immuno-electrophoresis (N. Axelsen et al., Chapter 2,).

In a still further aspect, the present invention relates to an enzyme preparation useful for the degradation of plant cell 20 wall components, said preparation being enriched in an enzyme exhibiting PME activity as described above.

The enzyme preparation having been enriched with an enzyme of the invention may e.g. be an enzyme preparation comprising 25 multiple enzymatic activities, in particular an enzyme preparation comprising multiple plant cell wall degrading enzymes such as Pectinex® or Pectinex Ultra SP® (Novo Nordisk A/S). In the present context, the term "enriched" is intended to indicate that the PME activity of the enzyme preparation has 30 been increased, e.g. with an enrichment factor of at least 1.1, conveniently due to addition of an enzyme of the invention prepared by the method described above.

Alternatively, the enzyme preparation enriched in an enzyme 35 exhibiting PME activity may be one which comprises an enzyme of the invention as the major enzymatic component, e.g. a mono-component enzyme preparation.

The enzyme preparation may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry preparation. For instance, the enzyme preparation may be in the form of a granulate or a microgranulate. The enzyme to
5 be included in the preparation may be stabilized in accordance with methods known in the art.

Based on its great activity on pectin containing material, typically of plant cell wall origin, a preferred use of the
10 enzyme preparation according to the invention is as an agent for degradation or modification of plant cell wall material or other pectin containing material.

The enzyme of the invention may advantageously be used together
15 with other enzymes, especially other pectin degrading enzymes when the enzyme is to be used in the processing of fruits, vegetables and other plant materials. Accordingly, the enzyme preparation of the invention may in addition to the PME comprise one or more other plant cell wall degrading enzymes
20 such as a polygalacturonase, pectin lyase, pectate lyase, arabinanase, xylanase, glucanase, galactanase, mannanase, rhamnogalacturonase, rhamnogalacturonan acetyl esterase or pectin acetylerase. The preparation may further contain one or more enzymes exhibiting exo-activity on the same substrates
25 as the above-mentioned endo-enzymes. In particular, the enzyme may be used in combination with polygalacturonase, pectate lyase or pectin lyase in pectin degradation.

Examples are given below of preferred uses of an enzyme
30 preparation of the invention comprising an enzyme exhibiting pectin methyl esterase activity, optionally in combination with one or more other enzymes. The dosage of the enzyme preparation of the invention and other conditions under which the preparation is used may be determined on the basis of methods
35 known in the art.

The enzyme preparation may advantageously be used for the treatment of pectin containing plant material, e.g. of vegetable or fruit origin, such as material obtained from soy

beans, sugar beets or apples, so as to reduce the viscosity and thus improve the processing or appearance of the plant material in question. The viscosity reduction may be obtained by treating the pectin-containing plant material with an enzyme
5 preparation of the invention under suitable conditions for full or partial degradation of the pectin-containing material.

The enzyme preparation may be used for de-pectinization and viscosity reduction in vegetable or fruit juice, especially in
10 apple or pear juice.

The enzyme preparation may be used in the treatment of mash from fruits and vegetables, for instance in the mash treatment of apples and pears for juice production, and in the mash
15 treatment of grapes for wine production.

The enzyme preparation may be used in the production of citrus juice, e.g. for partial or complete degradation of the pulp present in the juice after pressing.
20

For the above uses it is preferred that the enzyme preparation in addition to PME comprises a polygalacturonase containing enzyme preparation.

25 By use of an enzyme preparation of the invention it is possible to regulate the consistency and appearance of processed fruit or vegetables. Thus, the consistency and appearance have been found to be a product of the actual combination of enzymes used for the processing, i.e. the nature
30 of the enzymes (especially pectin degrading enzyme(s)) with which the pectin methyl esterase of the invention is combined.

Examples of products with specific properties which may be produced by use of an enzyme preparation of the invention
35 include clear juice from apples, pears or berries, cloud stable juice from apples, pears, berries, citrus, or tomatoes, and purees from carrots and tomatoes.

From the foregoing disclosure it will be apparent that the

PME of the invention may be produced as a single component essentially free from other enzyme activities such as polygalacturonase and/or pectin lyase activity normally found to be present in commercially available pectinesterase
5 containing pectinolytic preparations.

On this basis the use of the PME of the invention is especially advantageous for purposes in which the action of such other enzyme activities are undesirable.

10

Examples of such purposes include the use of the pectin methylesterase for full or partial demethylation of pectin in processed or non-processed fruits and vegetables.

15 The partial demethylation is, e.g., important when an improved firmness of fruits or vegetables is desirable. Thus, firmness is often reduced during processing (e.g. canning and pasteurization). By use of a controlled amount of a PME of the invention a partial demethylation of pectin present in the
20 surface of fruits and vegetables may be obtained and the resulting partially demethylated pectin may crosslink with, e.g., divalent ions such as calcium, whereby a more firm surface of the fruits or vegetables may be formed. Accordingly, the PME of the invention may be used for improving the firmness
25 of, e.g., beans, peas and sliced fruits such as pears and apples.

Another example of the purposes is demethylation of pectin, e.g. from citrus, apple, sunflower and/or sugar beet. The PME
30 can be used to produce low methylated pectin (defined as pectins where under 50% of the galacturonic acids are methylated) from high methylated pectin (defined as pectin where over 50% of the galacturonic acids are methylated). Conventionally, such demethylation has been carried out by
35 alkaline or acid demethylation which has the drawback, among others, that the demethylation needs to be carefully controlled in order to avoid depolymerization of the pectin backbone and thereby a severe reduction of the functional properties of the demethylated pectin.

By use of a PME of the invention the above described depolymerization is avoided and the demethylation is performed under milder conditions and within a reasonable temperature (15-50°C) and time level (10 min.- 2 hours).

5

Furthermore, the pectin esterase can be used to obtain an *in situ* viscosity increase or gel formation in various vegetable or fruit based products, when added together (simultaneously or non-simultaneously) with medium or high methylated pectin. The
10 resulting low methylated pectin will, in the presence of, e.g., divalent ions, form a more viscous liquid or a gel. Alternatively, the natural content of pectin may be demethylated by use of the enzyme, whereby the addition of pectin may be reduced or even avoided.

15

By use of this process it is possible to avoid or reduce the amount of pectin to be added. This is advantageous in that the addition of, e.g., low-methylated pectin may give rise to gelation during mixing which again may result in an incomplete
20 mixing being obtained. Furthermore, low-methylated pectin may be difficult to dissolve in water.

The use of PME for viscosity increase or gel formation eliminates or reduces the addition of other stabilizing or
25 gelling agents in, e.g., jam and ketchup. Thus, the demethoxylation of the natural content of pectin, which is induced by the enzyme, may, upon reaction with the natural content of metal ions, be sufficient for a satisfactory viscosity increase or gelformation to take place.

30

For the viscosity increase the amount of pectin methyl esterase to be added is typically in the range 0.1-100 PMEU per g of pectin, particularly 1-10 PMEU per g. The PME activity (PMEU) is defined in the Materials and Methods section below.

35

The PME of the invention can alone or together with other enzymes be used to improve the digestibility of pectin containing animal feed, e.g. feed prepared from soya beans,

sugar beets or rape seeds. For this purpose, an enzyme preparation of the invention is added to the feed.

The pectin esterase activity can together with other enzymes
5 be used to produce monogalacturonic acid or galacturonic acid containing oligosaccharides from pectin-containing material such as sugar beet pulp in accordance with well-known methods. Monogalacturonic acid may be used for production of galactaric acid or for production of fatty acid and fatty alcohol esters
10 and/or ethers of galacturonic acid. Galacturonic containing oligosaccharides may be used as additives for human food or animal feed.

Furthermore, the PME can in combination with other enzymes be
15 used for the removal of pectic substances from plant fibres, which removal is essential, e.g. in the production of textile fibres or other cellulosic materials. For this purpose plant fibre material is treated with a suitable amount of the PME of the invention under suitable conditions for obtaining full or
20 partial degradation of pectic substances associated with the plant fibre material.

The invention is further described in the accompanying drawing in which

25

Fig. 1 is a restriction map of plasmid pYHD17,

Fig. 2 a restriction map of plasmid pHD 414,

30 Fig. 3 a pH optimum curve,

Fig. 4 a temperature optimum curve,

Fig. 5 a pH stability curve,

35

Fig. 6 a temperature stability curve, and

Fig. 7 the $[S]/V$ versus $[S]$ curve used for K_m and spec. act. determination.

The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

5

EXAMPLES

Materials and Methods

10 **Donor organism:** mRNA was isolated from *Aspergillus aculeatus*, CBS 101.43, grown in a soy-containing fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C.

15

Yeast strains: The *Saccharomyces cerevisiae* strain used was YNG231 (MAT alpha, leu2, ura3-52, his4-539, pep4-delta 1, cir+) or JG169 (MAT α ; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-113; prc1::HIS3; prb1::LEU2; cir+).

20

Construction of an expression plasmid: The commercially available plasmid pYES II (Invitrogen) was cut with SpeI, filled in with Klenow DNA polymerase + dNTP and cut with ClaI. The DNA was size fractionated on an agarose gel, and a fragment
25 of about 2000 bp was purified by electroelution. The same plasmid was cut with ClaI/PvuII, and a fragment of about 3400 bp was purified by electroelution. The two fragments were ligated to a blunt-ended SphI/EcoRI fragment containing the yeast TPI promoter. This fragment was isolated from a plasmid
30 in which the TPI promoter from *S. cerevisiae* (cf. T. Albers and G. Kawasaki, J. Mol. Appl. Genet. 1, 1982, pp. 419-434) was slightly modified: an internal SphI site was removed by deleting the four bp constituting the core of this site. Furthermore, redundant sequences upstream of the promoter were
35 removed by Ball exonuclease treatment followed by addition of a SphI linker. Finally, an EcoRI linker was added at position -10. After these modifications, the promoter is included in a SphI-EcoRI fragment. Its efficiency compared to the original

promoter appears to be unaffected by the modifications. The resulting plasmid pYHD17 is shown in Fig. 1.

Preparation of RNase-free glassware, tips and solutions: All
5 glassware used in RNA isolations was baked at + 220 °C for at
least 12 h. Eppendorf tubes, pipet tips and plastic columns
were treated in 0.1 % diethylpyrocarbonate (DEPC) in EtOH for
12 h, and autoclaved. All buffers and water (except Tris-
containing buffers) were treated with 0.1 % DEPC for 12 h at 37
10 °C, and autoclaved.

Extraction of total RNA: The total RNA was prepared by
extraction with guanidinium thiocyanate followed by
ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin et
15 al., 1979) using the following modifications. The frozen
mycelia were ground in liquid N₂ to fine powder with a mortar
and a pestle, followed by grinding in a precooled coffee mill,
and immediately suspended in 5 vols of RNA extraction buffer (4
M GuSCN, 0.5 % Na-laurylsarcosine, 25 mM Na-citrate, pH 7.0,
20 0.1 M β-mercaptoethanol). The mixture was stirred for 30 min.
at RT° and centrifuged (30 min., 5000 rpm, RT°, Heraeus
Megafuge 1.0 R) to pellet the cell debris. The supernatant was
collected, carefully layered onto a 5.7 M CsCl cushion (5.7 M
CsCl, 0.1 M EDTA, pH 7.5, 0.1 % DEPC; autoclaved prior to use)
25 using 26.5 ml supernatant per 12.0 ml CsCl cushion, and
centrifuged to obtain the total RNA (Beckman, SW 28 rotor, 25
000 rpm, RT°, 24h). After centrifugation the supernatant was
carefully removed and the bottom of the tube containing the RNA
pellet was cut off and rinsed with 70 % EtOH. The total RNA
30 pellet was transferred into an Eppendorf tube, suspended in 500
μl TE, pH 7.6 (if difficult, heat occasionally for 5 min at 65
°C), phenol extracted and precipitated with ethanol for 12 h at
- 20 °C (2.5 vols EtOH, 0.1 vol 3M NaAc, pH 5.2). The RNA was
collected by centrifugation, washed in 70 % EtOH, and
35 resuspended in a minimum volume of DEPC-DIW. The RNA
concentration was determined by measuring OD_{260/280}.

Isolation of poly(A)⁺RNA: The poly(A)⁺RNAs were isolated by
oligo(dT)-cellulose affinity chromatography (Aviv & Leder,

1972). Typically, 0.2 g of oligo(dT) cellulose (Boehringer Mannheim) was preswollen in 10 ml of 1 x column loading buffer (20 mM Tris-Cl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1 % SDS), loaded onto a DEPC-treated, plugged plastic column (Poly Prep 5 Chromatography Column, Bio Rad), and equilibrated with 20 ml 1 x loading buffer. The total RNA was heated at 65 °C for 8 min., quenched on ice for 5 min, and after addition of 1 vol 2 x column loading buffer to the RNA sample loaded onto the column. The eluate was collected and reloaded 2-3 times by 10 heating the sample as above and quenching on ice prior to each loading. The oligo(dT) column was washed with 10 vols of 1 x loading buffer, then with 3 vols of medium salt buffer (20 mM Tris-Cl, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 0.1 % SDS), followed by elution of the poly(A)⁺ RNA with 3 vols of elution buffer (10 15 mM Tris-Cl, pH 7.6, 1 mM EDTA, 0.05 % SDS) preheated to + 65 °C, by collecting 500 µl fractions. The OD₂₆₀ was read for each collected fraction, and the mRNA containing fractions were pooled and ethanol precipitated at - 20 °C for 12 h. The poly(A)⁺ RNA was collected by centrifugation, resuspended in 20 DEPC-DIW and stored in 5-10 µg aliquots at - 80 °C.

Northern blot analysis: The poly(A)⁺ RNAs (5 µg/sample) from various mycelia were electrophoresed in 1.2 agarose-2.2 M formaldehyde gels (Sambrook et al., 1989) and blotted to nylon 25 membranes (Hybond-N, Amersham) with 10 x SSC (Sambrook et al., 1989) as transfer buffer. Three random-primed (Feinberg & Vogelstein, 1983) ³²P-labeled cDNA probes were used in individual hybridizations: 1) a 1.3 kb Not I-Spe I fragment for polygalacturonase I from *A. aculeatus* (described in Danish 30 Patent Application DK 1545/92), 2) a 1.3 kb Not I-Spe I fragment encoding endoglucanase I from *A. aculeatus* (described in DK 0419/92) and 3) a 1.2 kb Eag I fragment coding for galactanase I from *A. aculeatus* (described in WO 92/13945). Northern hybridizations were carried out in 5 x SSC (Sambrook 35 et al., 1989), 5 x Denhardt's solution (Sambrook et al., 1989), 0.5 % SDS (w/v) and 100 µg/ml denatured salmon sperm DNA with a probe concentration of ca. 2 ng/ml for 16 h at 65 °C followed by washes in 5 x SSC at 65 °C (2 x 15 min), 2 x SSC, 0.5 % SDS (1 x 30 min), 0.2 x SSC, 0.5 % SDS (1 x 30 min), and 5 x SSC (2

x 15 min). After autoradiography at - 80 °C for 12 h, the probe # 1 was removed from the filter according to the manufacturer's instructions and rehybridized with probe #2, and eventually with probe #3. The RNA ladder from Bethesda Research Laboratories was used as a size marker.

cDNA synthesis:

First strand synthesis: Double-stranded cDNA was synthesized from 5 µg of *A. aculeatus* poly(A)⁺ RNA by the RNase H method (Gubler & Hoffman 1983, Sambrook et al., 1989) using the hair-pin modification. The poly(A)⁺RNA (5 µg in 5 µl of DEPC-treated water) was heated at 70 °C for 8 min., quenched on ice, and combined in a final volume of 50 µl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM each dNTP (Pharmacia), 40 units of human placental ribonuclease inhibitor (RNasin, Promega), 10 µg of oligo(dT)₁₂₋₁₈ primer (Pharmacia) and 1000 units of SuperScript II RNase H- reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45 °C for 1 h.

Second strand synthesis: After synthesis 30 µl of 10 mM Tris-Cl, pH 7.5, 1 mM EDTA was added, and the mRNA:cDNA hybrids were ethanol precipitated for 12 h at - 20 °C by addition of 40 µg glycogen carrier (Boehringer Mannheim) 0.2 vols 10 M NH₄Ac and 2.5 vols 96 % EtOH. The hybrids were recovered by centrifugation, washed in 70 % EtOH, air dried and resuspended in 250 µl of second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM MgCl₂, 10 mM (NH₄)₂SO₄, 16 µM βNAD⁺) containing 100 µM each dNTP, 44 units of *E. coli* DNA polymerase I (Amersham), 6.25 units of RNase H (Bethesda Research Laboratories) and 10.5 units of *E. coli* DNA ligase (New England Biolabs). Second strand cDNA synthesis was performed by incubating the reaction tube at 16 °C for 3 h, and the reaction was stopped by addition of EDTA to 20 mM final concentration followed by phenol extraction.

Mung bean nuclease treatment: The double-stranded (ds) cDNA was ethanol precipitated at - 20 °C for 12 h by addition of 2 vols of 96 % EtOH, 0.1 vol 3 M NaAc, pH 5.2, recovered by centrifugation, washed in 70 % EtOH, dried (SpeedVac), and
5 resuspended in 30 µl of Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM ZnSO₄, 0.35 mM DTT, 2 % glycerol) containing 36 units of Mung bean nuclease (Bethesda Research Laboratories). The single-stranded hair-pin DNA was clipped by incubating the reaction at 30 °C for 30 min, followed by
10 addition of 70 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction, and ethanol precipitation with 2 vols of 96 % EtOH and 0.1 vol 3M NaAc, pH 5.2 at - 20 °C for 12 h.

Blunt-ending with T4 DNA polymerase: The ds cDNA was blunt-
15 ended with T4 DNA polymerase in 50 µl of T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM each dNTP and 7.5 units of T4 DNA polymerase (Invitrogen) by incubating the reaction mixture at + 37 °C for 15 min. The reaction was stopped by addition of
20 EDTA to 20 mM final concentration, followed by phenol extraction and ethanol precipitation.

Adaptor ligation and size selection: After the fill-in reaction the cDNA was ligated to non-palindromic BstX I adaptors (1
25 µg/µl, Invitrogen) in 30 µl of ligation buffer (50 mM Tris-Cl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 µg/ml bovine serum albumin) containing 600 pmol BstX I adaptors and 5 units of T4 ligase (Invitrogen) by incubating the reaction mix at + 16 °C for 12 h. The reaction was stopped by heating at + 70 °C for 5
30 min, and the adapted cDNA was size-fractionated by agarose gel electrophoresis (0.8 % HSB-agarose, FMC) to separate unligated adaptors and small cDNAs. The cDNA was size-selected with a cut-off at 0.7 kb, and the cDNA was electroeluted from the agarose gel in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA for 1 h at 100
35 volts, phenol extracted and ethanol precipitated at - 20 °C for 12 h as above.

Construction of cDNA libraries: The adapted, ds cDNA was recovered by centrifugation, washed in 70 % EtOH and

resuspended in 25 ml DIW. Prior to large-scale library ligation, four test ligations were carried out in 10 μ l of ligation buffer (same as above) each containing 1 μ l ds cDNA (reaction tubes #1 - #3), 2 units of T4 ligase (Invitrogen) and 5 50 ng (tube #1), 100 ng (tube #2) and 200 ng (tubes #3 and #4) Bst XI cleaved yeast expression vector either pYES 2.0 vector (Invitrogen or yHD13). The ligation reactions were performed by incubation at + 16 °C for 12 h, heated at 70 °C for 5 min, and 1 μ l of each ligation electroporated (200 Ω , 2.5 kV, 25 μ F) to 10 40 μ l competent E. coli 1061 cells (OD600 = 0.9 in 1 liter LB-broth, washed twice in cold DIW, once in 20 ml of 10 % glycerol, resuspended in 2 ml 10 % glycerol). After addition of 1 ml SOC to each transformation mix, the cells were grown at + 37 °C for 1 h , 50 μ l plated on LB + ampicillin plates (100 15 μ g/ml) and grown at + 37 °C for 12h.

Using the optimal conditions a large-scale ligation was set up in 40 μ l of ligation buffer containing 9 units of T4 ligase, and the reaction was incubated at + 16 °C for 12 h. The 20 ligation reaction was stopped by heating at 70 °C for 5 min, ethanol precipitated at - 20 °C for 12 h, recovered by centrifugation and resuspended in 10 μ l DIW. One μ l aliquots were transformed into electrocompetent E. coli 1061 cells using the same electroporation conditions as above, and the 25 transformed cells were titered and the library plated on LB + ampicillin plates with 5000-7000 c.f.u./plate. To each plate was added 3 ml of medium. The bacteria were scraped off, 1 ml glycerol was added and stored at -80°C as pools. The remaining 2 ml were used for DNA isolation. If the amount of DNA was 30 insufficient , to give the required number of yeast transformants, large scale DNA was prepared from 500 ml medium (TB) inoculated with 50 μ l of -80°C bacterial stock propagated overnight.

35 **Construction of yeast libraries:** To ensure that all the bacterial clones were tested in yeast, a number of yeast transformants 5 times larger than the number of bacterial clones in the original pools was set as the limit.

One μ l aliquots of purified plasmid DNA (100 ng/ μ l) from individual pools were electroporated (200 Ω , 1.5 kV, 25 μ F) into 40 μ l competent *S. cerevisiae* JG 169 cells (OD600 = 1.5 in 500 ml YPD, washed twice in cold DIW, once in cold 1 M sorbitol, resuspended in 0.5 ml 1 M sorbitol, Becker & Guarante, 1991). After addition of 1 ml 1M cold sorbitol, 80 μ l aliquots were plated on SC + glucose - uracil to give 250-400 c.f.u./plate and incubated at 30 °C for 3 - 5 days.

10 **Construction of an *Aspergillus* expression vector:** the vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). In contrast to this plasmid, pHD 414 has a string of unique restriction sites between the promoter and the terminator. The plasmid was constructed by removal of an
15 approximately 200 bp long fragment (containing undesirable RE sites) at the 3'end of the terminator, and subsequent removal of an approximately 250 bp long fragment at the 5'end of the promoter, also containing undesirable sites. The 200 bp region was removed by cleavage with NarI (positioned in the pUC
20 vector) and XbaI (just 3' to the terminator), subsequent filling in the generated ends with Klenow DNA polymerase +dNTP, purification of the vector fragment on gel and religation of the vector fragment. This plasmid was called pHD413. pHD413 was cut with StuI (positioned in the 5'end of the promoter) and
25 PvuII (in the pUC vector), fractionated on gel and religated. The plasmid pHD 414 is shown in Fig. 2.

Media:

YPD: 10 g yeast extract, 20 g peptone, H₂O to 810 ml.
30 Autoclaved, 90 ml 20% glucose (sterile filtered) added.

10 x Basal salt: 66.8 g yeast nitrogen base, 100 g succinic acid, 60 g NaOH, H₂O ad 1000 ml, sterile filtered.

35 SC-URA: 90 ml 10 x Basal salt, 22.5 ml 20 % casamino acids, 9 ml 1% tryptophan, H₂O ad 806 ml, autoclaved, 3.6 ml 5% threonine and 90 ml 20% glucose or 20% galactose added.

SC-H broth: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan. Autoclaved for 20 min. at 121°C. After autoclaving, 10 ml of a 30% galactose solution, 5 ml of a 30% glucose solution and 0.4 ml of a 5% threonine solution were added per 100 ml medium.

SC-H agar: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan, and 20 g/l agar (Bacto). Autoclaved for 20 min. at 121°C. After autoclaving, 55 ml of a 22% galactose solution and 1.8 ml of a 5% threonine solution were added per 450 ml agar.

15 YNB-1 agar: 3.3 g/l KH_2PO_4 , 16.7 g/l agar, pH adjusted to 7. Autoclaved for 20 min. at 121°C. After autoclaving, 25 ml of a 13.6% yeast nitrogen base without amino acids, 25 ml of a 40% glucose solution, 1.5 ml of a 1% L-leucine solution and 1.5 ml of a 1% histidine solution were added per 450 ml agar.

20

YNB-1 broth: Composition as YNB-1 agar, but without the agar.

FG-4-Agar: 35 g/L agar, 30 g/L Soy bean meal, 15 g/L maltodextrin (Glucidex 6), 5 g/L Bacto pepton, pH 7. Autoclaved 40 min at 121°C

FG-4 medium: 30 g/L Soy bean meal, 15 g/L maltodextrin (Glucidex 6), 5 g/L Bacto peptone. Autoclaved 40 min at 121°C.

30 MDU-2 medium: 45 g/L maltose, 1 g/L $\text{MgSO}_4 - 7 \text{ H}_2\text{O}$, 1 g/L NaCl, 2g/L K_2SO_4 , 12 g/L KH_2PO_4 , 0.1 ml/L Pluronic 61 L, 0.5 ml/L Trace metal solution. pH 5.0. Autoclaved 20 min at 121°C. 15 ml/L 50% sterile filtered urea is added after autoclaving.

35 Pectin overlay gel: 1% HSB agarose, 1% pectin (DE 75%) in a buffer with an appropriate pH. The gel was boiled and then cooled to 55°C before the overlay was poured onto agar plates.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*
(general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) is inoculated with spores of *A. oryzae* or *A. niger* and incubated with shaking at 37°C for about 2 days. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M MgSO₄. The mycelium is suspended in 15 ml of 1.2 M MgSO₄. 10 mM NaH₂PO₄, pH = 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym[®] 234, batch 1687 is added. After 5 minutes 1 ml of 12 mg/ml BSA (Sigma type H25) is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37°C until a large number of protoplasts is visible in a sample inspected under the microscope.

15

The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlaid with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation is performed for 15 minutes at 100 g and the protoplasts are collected from the top of the MgSO₄ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5. 10 mM CaCl₂) are added to the protoplast suspension and the mixture is centrifugated for 5 minutes at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC. Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576). 10 mM CaCl₂ and 10 mM Tris-HCl, pH = 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on the appropriate plates. Protoplasts are spread on minimal plates (Cove Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen

source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a
5 defined transformant.

Fed batch fermentation

Fed batch fermentation was performed in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and
10 yeast extract. The fed batch fermentation was performed by innoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 5.0 and 34°C the continuous supply of additional carbon and
15 nitrogen sources were initiated. The carbon source was kept as the limiting factor and it was secured that oxygen was present in excess. The fed batch cultivation was continued for 4 days, after which the enzymes could be recovered by centrifugation, ultrafiltration, clear filtration and germ filtration.

20

Purification of the enzyme

The recombinant enzyme from *A. oryzae* was purified as follows: A culture supernatant was harvested after 5 days of culture, and centrifuged, sterile filtered, and concentrated on a 20 kDa
25 ultrafiltration device to approximately 20% dry matter. 40 ml of this concentrate (containing 80-120 mg rPME) were diluted 10 times in 20 mM Tris pH 8.0 and applied to a HR 16/20 Q-Sepharose fast flow column (Pharmacia, Sweden) at 1.5 ml/min, and eluted with a linear NaCl gradient (from 0 to 0.6M NaCl) at
30 approximately 0.4 M NaCl. The fractions containing pectin methyl esterase activity were pooled and ultrafiltrated into 20 mM citrate pH 3.0 and loaded on a HR 16/20 S-Sepharose fast flow column (Pharmacia, Sweden) at 1.5 ml/min, and eluted with a linear NaCl gradient at approximately 0.2 M NaCl. The
35 fractions containing PME activity were ultrafiltrated in water and used for characterization as described below. Protein concentrations in the fractions were determined by the Bio Rad protein assay (Bio Rad, USA).

Characterization of an enzyme of the invention:Electrophoresis

SDS-PAGE electrophoresis was performed in a Mini-Leak 4 electrophoresis unit (Kem-En-Tec, Denmark) as a modified version of the Laemli procedure (Laemmli 1970). Isoelectric focusing was carried out on Ampholine PAG plates pH 3.5 - 9.5 (Pharmacia, Sweden) on a Multiphor electrophoresis unit according to the manufactures instructions. Gels were either silverstained essentially as described in (Merrild, Switzer et al. 1979) or coomassie stained according to (Matsudaira 1989).

pH stat measurements

The equipment used was a titrator TTT80; autoburette ABU80; Titrigraph module REA 160; pH stat unit REA 270 all manufactured by Radiometer, Copenhagen, Denmark. The incubations were done in a 15 ml reactor cell connected to a thermostat and a magnetic stirrer. The reactor was filled with 15 ml 0.2% substrate solution in water, the substrate being apple pectin with a 75% of esterification produced from apple pectin manufactured by Obipektin AG. pH was adjusted to 4.5 with NaOH or HCl (for determination of pH optimum, pH was adjusted to other values as described below), and the temperature of the reaction cell was maintained at 30°C (except for determination of temperature optimum, as described below). After the enzyme sample had been injected in the reactor, the cell was left stirring for 1 minute, whereafter the reaction was measured over 1.5 minutes. The pH stat injected 10 mM NaOH in the reactor cell to maintain constant pH, and the number of hydrolyzed methyl ester linkage (hmel) is directly proportional to the amount of NaOH injected into the reactor according to the following equation:

$$n(\text{hmel}) = n(\text{NaOH}) (1 + [\text{H}^+]/K_a)$$

) The pK_a of galacturonic acid is approximately 3.5 at 30°C.

35

Determination of pH optimum

The experiments were conducted at pH values varying from 2.5-6.0. 1.5 µg of purified enzyme in a volume of 50 ml were added to the solution and measurements carried out as described

above. The activity was expressed as percentage of the maximal activity (pH 4.55).

Determination of pH stability

5 For determination of pH stability, 1.5 μ g of enzyme in a volume of 50 ml were added to 10 ml 0.1% substrate solution in the reactor cell, and the pH adjusted to a value between 2.5 and 8. After 1 hour incubation at 30°C, 5 ml 0.4% substrate solution were added and pH adjusted to 4.5. Subsequently activity
10 measurements were carried out as described above.

Determination of temperature optimum

The experiments were conducted at temperature values varying from 25-62°C. 1.5 μ g of purified enzyme in a volume of 50 ml
15 were added to the solution and measurements carried out as described above. The activity was expressed as percentage of the maximal activity (43°C).

Determination of temperature stability

20 The enzyme samples were incubated at various temperatures between 30° and 80° for 0.1 and 2 hours, after which the activity was measured as described above.

Determination of pectin methylesterase activity

25 1 unit of pectin methyl esterase activity (PMEU) is defined as the amount of pectin methyl esterase which hydrolyzes 1 μ mole of pectin methyl ester per minute with citrus pectin (72 % methylation) as the substrate at 0.5 % by weight substrate
30 concentration at pH 4.8 and 22°C.

EXAMPLE 1

35 A library from *A. aculeatus* consisting of approx. 1.5×10^6 individual clones in 150 pools was constructed.

DNA was isolated from 20 individual clones from the library and subjected to analysis for cDNA insertion. The insertion

frequency was found to be >90 % and the average insert size was approximately 1400bp.

DNA from some of the pools was transformed into yeast, and 50-
5 100 plates containing 200-500 yeast colonics were obtained from each pool. After 3-5 days of growth, the agar plates were replica plated onto several sets of agar plates. One set of plates was then incubated for 2-4 days at 30°C and overlaid with a pectin overlay gel for detection of pectinolytic
10 activity. After incubation overnight at 30°C, 10-15 ml of a 1% solution of MTAB (mixed alkyltrimethylammonium bromide) was poured onto the overlay and removed after 1 hour. PME positive colonies were identified as colonies surrounded by a white halo.

15

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the PME-producing colonies identified.

20 **Characterization of positive clones:** The positive clones were obtained as single colonies, the cDNA inserts were amplified directly from the yeast colony using biotinylated polylinker primers, purified by magnetic beads (Dynabead M-280, Dynal) system and characterized individually by sequencing the 5'-end
25 of each cDNA clone using the chain-termination method (Sanger et al., 1977) and the Sequenase system (United States Biochemical). The cDNA sequence encoding the enzyme is shown in SEQ ID no. 1.

30 **Isolation of a cDNA gene for expression in Aspergillus:**

One or more of the PME-producing colonies were inoculated into 20 ml YNB-1 broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

35

The cells were resuspended in 1 ml 0.9 M sorbitol, 0.1 M EDTA, pH 7.5. The pellet was transferred to an Eppendorf tube, and spun for 30 seconds at full speed. The cells were resuspended in 0.4 ml 0.9 M sorbitol, 0.1 M EDTA, 14 mM β -mercaptoethanol.

100 μ l 2 mg/ml Zymolase was added, and the suspension was incubated at 37°C for 30 minutes and spun for 30 seconds. The pellet (spheroplasts) was resuspended in 0.4 ml TE. 90 μ l of (1.5 ml 0.5 M EDTA pH 8.0, 0.6 ml 2 M Tris-Cl pH 8.0, 0.6 ml 5 10% SDS) was added, and the suspension was incubated at 65°C for 30 minutes. 80 μ l 5 M KOAc was added, and the suspension was incubated on ice for at least 60 minutes and spun for 15 minutes at full speed. The supernatant was transferred to a fresh tube which was filled with EtOH (room temp.) followed by 10 thorough but gentle mixing and spinning for 30 seconds. The pellet was washed with cold 70% EtOH, spun for 30 seconds and dried at room temperature. The pellet was resuspended in 50 μ l TE and spun for 15 minutes. The supernatant was transferred to a fresh tube. 2.5 μ l 10 mg/ml RNase was added, followed by 15 incubation at 37°C for 30 minutes and addition of 500 μ l isopropanol with gentle mixing. The mixture was spun for 30 seconds, and the supernatant was removed. The pellet was rinsed with cold 96% EtOH and dried at room temperature. The DNA was dissolved in 50 μ l water to a final concentration of 20 approximately 100 μ l/ml.

The DNA was transformed into *E. coli* by standard procedures. Two *E. coli* colonies were isolated from each of the transformations and analysed with the restriction enzymes 25 HindIII and XbaI which excised the DNA insert. DNA from one of these clones was retransformed into yeast strain JG169.

The DNA sequences of several of the positive clones were determined. The entire DNA sequence of a PME is shown in SEQ ID 30 No. 1, a partial DNA sequence is shown in SEQ ID No. 3.

EXAMPLE 2

35 In order to express the genes in *Aspergillus*, cDNA is isolated from one or more representatives of each family by digestion with HindIII/XbaI or other appropriate restriction enzymes, size fractionation on a gel and purification and subsequently ligated to pHD414, resulting in the plasmid pA1PE1.2. After

amplification in *E. coli*, the plasmids are transformed into *A. oryzae* or *A. niger* according to the general procedure described above.

5 Test of *A. oryzae* transformants

Each of the transformants was inoculated in the center of a Petri dish with FG-4 agar. After 5 days of incubation at 30°C 4 mm diameter plugs were removed from the center of the colonies by a corkscrew. The plugs were embedded in a pectin
10 overlay gel and incubated overnight at 40°C. The PME activity was identified as described above. Some of the transformants had halos which were significantly larger than the *A. oryzae* background. This demonstrates efficient expression of PME in *A. oryzae*. The 8 transformants with the highest PME activity were
15 selected and inoculated and maintained on YPG-agar.

Each of the 8 selected transformants were inoculated from YPG-agar slants on 500 ml shake flask with FG-4 and MDU-2 media. After 3-5 days of fermentation with sufficient agitation to
20 ensure good aeration, the culture broths were centrifuged for 10 minutes at 2000 g and the supernatants were analyzed.

A volume of 15 μ l of each supernatant was applied to 4 mm diameter holes punched out in a pectin overlay gel (25 ml in
25 a 13 cm diameter Petri dish). The PME activity was identified by the formation of a white halo on incubation.

Subsequently, the PME was produced by fed batch fermentation of *A. oryzae* expressing the enzyme as described in Materials and
30 Methods above.

EXAMPLE 3

35 Purification and characterization of a cloned recombinant PME of the invention

PME hydrolyzes the ester-linkage between methanol and galacturonic acid in esterified pectin. Hence, the action of

the enzyme can be measured by the decrease in pH which happens concomitant with the formation of free acid groups. This analysis method has been used for determination of K_m , V_{max} , pH & Temperature optimum of a PME of the invention as well as
5 determination of how temperature, pH and substrate concentration affects the activity of the enzyme.

Purification of the enzyme

The purification of a recombinant enzyme from *A. oryzae*
10 produced as described above was purified and characterized by the methods described in the Materials and Methods section above. The following results were obtained:

The molecular weight of the enzyme was determined to 43 kD by
15 SDS-PAGE. This is higher than the calculated molecular weight of the enzyme (approx. 35 kD) indicating that the enzyme may be glycosylated to a significant extent. The isoelectric point was determined to 3.8 by isoelectric focusing, which is slightly lower than the calculated value (4.1). This may also indicate
20 that the enzyme undergoes a significant post-translational processing (e.g. glycosylation).

The pH optimum was measured to approximately pH 4.5 (Fig. 3) and the temperature optimum to 45°C (Fig. 4). the activity
25 drops sharply at pH above 4.5 and temperatures above 50°C, whereas the drop in activity at lower temperatures and pH was less pronounced. The special method of measuring pH stability precludes an exact measurement of pH sensitivity of the enzyme, but the PME of the invention appears to be most stable at
30 neutral pH, i.e. above 5.5-6 such as within pH 6-8 (Fig. 5). The enzyme was relatively sensitive to elevated temperatures, i.e. temperatures above 50°C. Already after 1 hour at 50°, almost 80% of the activity has been lost (Fig. 6).

35 Finally, the reaction speed, V_{max} , and K_M was determined for the enzyme by varying the substrate concentration during incubation, and plot the results in a Hanes plot, where $[S]/V = [S] 1/V_{max} + K_m/V_{max}$ (Fig. 7). K_m was measured to 2.8% on 75% esterified apple pectin (apple pectin, with a 75% degree of

esterification). This is a relatively high value, which indicates a relatively low affinity to the substrate. The maximal velocity of the enzyme, V_{max} , was determined to 8.6 $\mu\text{mol}/\text{min}$, and the specific activity was determined to 5.5 $\text{mmol}/(\text{min}/\text{mg})$.

When using citruc pectin (DE=70-72%) as a substrate and performing the assay at a temperature of 22°C, the K_m was determined to be 0.5%, the V_{max} to 6.0 $\mu\text{mol}/\text{min}$, and the specific activity was determined to 239 $\mu\text{mol}/(\text{min}/\text{mg})$. Furthermore, under these conditions the pH optimum was determined to approx. 4.8.

The enzyme was not able to hydrolyze acetyl-ester groups from neither acetylated polysaccharides, nor the synthetic acetyl-esterase substrate p-nitro-phenol-acetate, demonstrating its substrate specificity. The specific activity on apple pectin of 5.5 $\text{mmol}/(\text{min}/\text{mg})$ indicates that the enzyme has a very high catalytic capacity.

As expected the enzyme has a significant synergistic effect on pectin degradation when used in combination with polygalacturonases. By incubation with an *A. aculeatus* polygalacturonase (PCT/DK93/00445) with or without PME of the invention it was found that polygalacturonase alone has only limited activity towards the pectin, PME alone has no effect on the degradation of the pectin, but the use of PME in combination with polygalacturonase significantly increases the substrate degrading capability of the polygalacturonase whereby oligomeric material is formed.

The acidic pH optimum of the enzyme indicates that it may be of great value in fruit juice processing where the pH frequently is below pH 5.0, but it may also be of use in the wine, feed and food industry, where pectin containing plant material is processed.

EXAMPLE 4

D methylation of pectin

5 40.0 g of pectin having a degree of esterification of 72% were diluted in 2000 mls, 80°C demineralized water. The pectin solution was tempered to 30°C, pH was adjusted to 4.5 with 0.25 M NaHCO₃.

10 34 PMEU/g pectin of PME produced as described above were added to 2000 mls of pectin solution. At the time 10, 30, 60, 120, 180 min., 50 mls samples were taken out and heat treated to inactivate the enzyme (100°C, 10 min.). Afterwards, the samples were analysed in accordance with the following method:

15

6.25 g of the 2% enzyme treated solutions was added and dissolved in 1.25 ml ethanol and 25 ml CO₂-free demineralized water. The amount of free carboxyl groups is determined by titration with 0.2 M NaOH to the equivalence point (pH 7.0) to
20 determine the nonesterified galacturonic acid units. A known excess of aqueous NaOH was added and saponification was allowed to take place for 1 hour. Then, an equivalent amount of HCl was added and the sample was again titrated to pH 7.0 to determine the amount of esterified galacturonic acid units. The degree of
25 esterification, DE%, was then calculated.

The results are apparent from the table below:

	Time,min.	0	10	30	60	120	180
30	DE%	(72)*	52	39	33	27	26

*) not measured.

EXAMPLE 5

Orange marmalade

Oranges were washed and minced in a meat chopping machine. The
5 fruit was added sugar in the level fruit/sugar: 5/1 based on
weight. Then the fruit mass was cooked for about 15 min and
cooled to -18°C.

The fruit mass was later diluted 1:1 by demineralized water,
10 tempered to 40°C and pectin methyl esterase prepared as
described above was added to 50 g samples in amounts of 17, 1.7
and 0.17 PMEU/g of pectin. The pH of the samples remained
unadjusted 3.5. The pectin was estimated to be 30% w/w of the
fruit. A control was also prepared, consisting of 17 PMEU/g of
15 pectin (inactivation 85°C for 3 minutes). The reaction time was
1 hour.

The samples were then cooled to 4°C over night and the
following day the hardness of the gels was evaluated at 4°C,
20 before and after a heat treatment (85°C for 3 min) of the
samples.

The measurement of the hardness of the gels was carried out by
using SMS Texture Analyser. The conditions of the texture
25 analysis were as follows:

Probe, diameter mm: 20
Penetration: 20%
Rate: 2 mm/sek

The hardness, N, appears from below:

	17 PMEU/g N	1.7 PMEU/g N	0.17 PMEU/g N	contro l N
5 Before inactiva tion	5.24	2.11	0.26	0.08
10 After inactiva tion	3.97	1.52	0.26	0.09

EXAMPLE 6

Tomato paste

15 Canned, peeled plum tomatoes added tomatojuice and citric acid, bought at the grossery store were used as a substrate for the following PME-trials.

Pectin methyl esterase prepared as described above was added to
20 a portion of 100 g in an amount of 5 PMEU/g tomato product, a control was added 5 PMEU of inactivated PME/g tomato product. The samples were mixed and left for 40°C for 30 minutes. The samples were then placed at 4°C until the next day and measured by SMS Texture Analyser at 17°C. The samples were heat treated
25 for 85°C in 3 min to inactivate the enzyme and after having adjusted the temperature to 17°C, the samples were again measured by SMS Texture Analyser.

The texure analyser measures the hardness, N. The probe is a 20
30 mm probe, penetration of the sample was 20%, the speed was 2 mm/sek.

pH of both the control and the test sample was 4.4.
The results are apparent from the table below.

	Hardness, N before inactivation	Hardness, N after inactivation
test sample	0.31	0.24
control	0.09	0.10

5

EXAMPLE 6**Pudding-Like Milk Dessert**

10 198 g of milk was heated to 80°C in a microwave oven. 2 g of rapid set HM pectin was added at 80°C by vigorous mixing. The milk was afterwards tempered to 30°C and pectin methyl esterase prepared as described above was added to 100 ml of the mixture in an amount of 5 PMEU/ml. A 100 ml control was also prepared
15 containing the same amount of inactivated enzyme (85°C in 3 min). The pH of the two samples was 6.3.

The test sample containing active enzyme gelled in 10 min while the control remained fluent.

20

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30 Cambridge University Press.

SEQUENCES

SEQ ID No. 1

- 5 *A. aculeatus* Pectin methylesterase DNA sequence. The start and stop codons (indicating the coding part of the DNA sequence) are marked in bold.

AGTCGATCATACACTCATATCAATCGCAAAAATGGTTAAATCAGTCTTGGCT
10 TCCGCTCTCTTCGCCGTGTCCGCACTGGCTGCCAGCCGTACCACGGCTCCCTCC
GGCGGATCGTCGTCGCCAAGTCTGGTGGTGA CTATACTATTGGTGA TGC
CATTGATGCTCTGAGCACCAGCACCACCGACACCCAAACCATTTTCATCGAG
GAGGGTACCTACGATGAGCAGGTCTACCTGCCTGCTATGACCGGCAAGGTCAT
CATCTACGGTCAAACCGAGAACCCGACTCCTACGCCGACAACCTGGTCACCA
15 TCACCCACGCCATCAGCTACGAGGATGCTGGTGAGAGCGATGATCTGACTGCT
ACCTTCCGCAACAAGGCTGTCCGCTCTCAGGTCTACAACCTCAACATTGCCAA
CACCTGTGGTCAGGCTTGCCACCAGGCTTTGGCCTTGTCGCCCTGGGCTGACCA
GCAGGGTTACTACGGCTGCAACTTCACTGGTTACCAGGATACCCTCCTCGCTCA
GACCGGTAACCAGCTCTACATCAACTCCTACATTGAGGGTGCTGTGCACTTCAT
20 CTTTGGCCAGCACGCTCGTGCTTGGTTCCAGAACGTCGACATCCGTGTGCTTGA
GGGTCCTACCTCTGCCTCCATCACCGCCAACGGCCGCTCCTCCGAGACTGACAC
CTCCTACTACGTGATCAACAAGTCGACCGTTGCTGCTAAGGAGGGCGACGACGT
TGCGGAAGGGACCTACTACCTTGGCCGCCCTGGTCCGAGTACGCCCCTGTCGTC
TTCCAGCAGACCAGCATGACCAACGTCATCAACTCCCTCGGCTGGACTGAGTGG
25 TCCACCTCCACCCCTAACACCGAGTACGTCACCTTCGGCGAGTACGCAAACCAA
CCGGCGCCGGCTCCGAGGGCACCCGCGCACGTCGCCGAGAAGCTGGATGCCAAGC
TCACCATCACGGATATCCTGGGCTCTGACTACACCAGCTGGGTCGATACCTCCT
ACTTCTAAGCTGGACGCATGAGTAGTTGTTAAGAATGCGTGAGATGGCTGTCAA
CCAACAGCAGAGCTGACCGTATGCGGTGCATATACTTTCTCTTCGTCAAATAATTTCCCT
30 TTGATAAAGCAATTTAATTGGCATGCACAGTCCATGCTTGACACAAA
AAAAAAAAAAAAAA

SEQ ID No. 2

The *A. aculeatus* pectin methylesterase sequence.

5

MVKSVLASATFAVSATAASRTTAPSGAIVVAKSGGDYTTIG
DAIDATSTSTTDTQTIFIEEGTYDEQVYTPAMTGKVIIYGQTEN
TDSYADNTVTITHAISYEDAGESDDTTATFRNKAVGSQVYNTN
IANTCGQACHQALALSAWADQQGYGNCNFTGYQDTTTAQTGN
QTYINSYIEGAVDFIFGQHARAWFQNVDIRVVEGPTSASITAN
GRSSETDTSYYVINKSTVAAKEGDDVAEGTYTGRPWSEYARV
VFQQTSMTNVINSTGWTEWSTSTPNTEYVTFGEYANQPAPAP
RAPAHVAEKTDAKTTITDITGSDYTSWVDTSYF

CLAIMS

1. An enzyme exhibiting pectin methylesterase activity which enzyme

5

a) is immunologically reactive with an antibody raised against a purified pectin methylesterase derived from *Aspergillus aculeatus*, CBS 101.43, and/or

10 b) is encoded by the coding part of the DNA sequence shown in SEQ ID No. 1 or an analogue of said sequence, and/or

c) has the amino acid sequence shown in SEQ ID No. 2 or a sequence which is at least 80% homologous thereto.

15

2. An enzyme exhibiting pectin methylesterase activity, which enzyme is encoded by a DNA sequence comprising the following partial sequence

20 TAAAGAGTCG ATCATACACT CATATCAATC GCAAAAATGG TTAAATCAGT
 CTTGGCTTCC GCTCTCTTCG CCGCGTCCGC ACTGGCTGCC AGCCGTACCA
 CGGCTCCCTC CGGCGCGATC GTCGTCGCCA AGTCTGGTGG TGACTATACC
 ACTATTGGTG ATGCCATTGA TGCTCTGAGC ACCAGCACCA CCGACACCCA
 AACCATTTTC ATCGAGGAG GGTACCTAC GATGAGCAGG TCTACCTGCC TGCTATGACC
 25 GGCAAGGTCA TCATCTACGT CAAACCGAGA ACACCGACTC CTACG

or a sequence homologous thereto encoding a polypeptide with pectin methylesterase activity.

30 3. An enzyme exhibiting pectin methylesterase activity, which enzyme is encoded by a DNA sequence comprising at least one of the following partial sequences

(a) TAAAGAGTCG ATCATACACT
 35 (b) CATATCAATC GCAAAAATGG
 (c) TTAAATCAGT CTTGGCTTCC
 (d) GCTCTCTTCG CCGCGTCCGC
 (e) ACTGGCTGCC AGCCGTACCA
 (f) CGGCTCCCTC CGGCGCGATC

- (g) GTCGTCGCCA AGTCTGGTGG
(h) TGACTATACC ACTATTGGTG
(i) ATGCCATTGA TGCTCTGAGC
(j) ACCAGCACCA CCGACACCCA
5 (k) AACCATTTTC ATCGAGGAG
(l) GGTACCTAC GATGAGCAGG
(m) TCTACCTGCC TGCTATGACC
(n) GGCAAGGTCA TCATCTACGT
(o) CAAACCGAGA ACACCGACTC CTACG

10

4. An enzyme according to any of claims 1-4, which has one or more of the following properties:
- a pH optimum of 4.5 as determined at 30°C using 75% esterified pectin as a substrate;
 - 15 - a temperature optimum of 45°C as determined at 30°C using 75% esterified pectin as a substrate,
 - a molecular weight of 43 kD determined by SDS-PAGE.

4. An enzyme according to any of claims 1-3, which is derivable
20 from a microorganism.

5. An enzyme according to claim 4, which is derivable from a filamentous fungus or a yeast.

- 25 6. An enzyme according to claim 5, which is derivable from a strain of *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium* or *Humicola*.

7. An enzyme according to claim 6, wherein the enzyme is
30 derivable from a strain of *Aspergillus*, in particular a strain of *Aspergillus aculeatus*, *Aspergillus niger* or *Aspergillus oryzae*.

8. An enzyme according to claim 7, which is encoded by the DNA
35 sequence isolated from a DNA library of *Aspergillus aculeatus*, CBS 101.43.

9. A DNA construct comprising a DNA sequence encoding an enzyme exhibiting pectin methyl esterase activity, which DNA sequence

- comprises or is comprised in the DNA sequence shown in SEQ ID No. 1 or in any of the DNA sequences defined in claims 2 and 3 or is an analogue of such sequence, which
- 5 i) hybridizes with an oligonucleotide probe prepared on the basis of the DNA sequence shown in any of the DNA sequences defined in claims 2 or 3, the DNA sequence shown in SEQ ID No. 1, or the amino acid sequence shown in SEQ ID No. 2,
- 10 ii) encodes an enzyme comprising an amino acid sequence being at least 80% homologous with the amino acid sequence shown in SEQ ID No. 2, and/or
- iii) encodes an enzyme which is immunologically cross-reactive
15 with the polypeptide comprising the amino acid sequence shown in SEQ ID No. 2.
10. A recombinant expression vector comprising the DNA construct according to claim 9 or a DNA sequence encoding an
20 enzyme according to any of claims 1-8.
11. A cell comprising a recombinant expression vector according to claim 10.
- 25 12. A cell according to claim 11, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.
13. A cell according to claim 12, wherein the cell belongs to
30 a strain of *Aspergillus*, in particular a strain of *Aspergillus niger* or *Aspergillus oryzae*.
14. A method of producing an enzyme exhibiting pectin methylesterase activity, the method comprising culturing a cell
35 according to any of claims 11-13 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

15. An enzyme preparation useful for the modification of plant cell wall components, said preparation being enriched in an enzyme exhibiting pectin methylesterase activity according to any of claims 1-8.

5

16. A preparation according to claim 15, which additionally comprises one or more other plant cell wall degrading enzymes such as a pectin lyase, pectate lyase, arabinanase, xylanase, glucanase, galactanase, mannanase, rhamnogalacturonase, 10 rhamnogalacturonan acetylerase, pectin acetylerase, polygalacturonase or pectin methylesterase.

17. Use of a pectin methylesterase according to any of claims 1-8 for improving the firmness of a pectin-containing material.

15

18. Use of a pectin methylesterase according to any of claims 1-8 for demethylation of pectin.

19. Use of a pectin methylesterase according to any of claims 20 1-8 for increasing the viscosity of a pectin-containing material.

20. The use according to claim 18, in which the pectin-containing material is a fruit or vegetable material.

25

21. The use according to claim 18, in which the pectin-containing material is milk comprising added pectin.

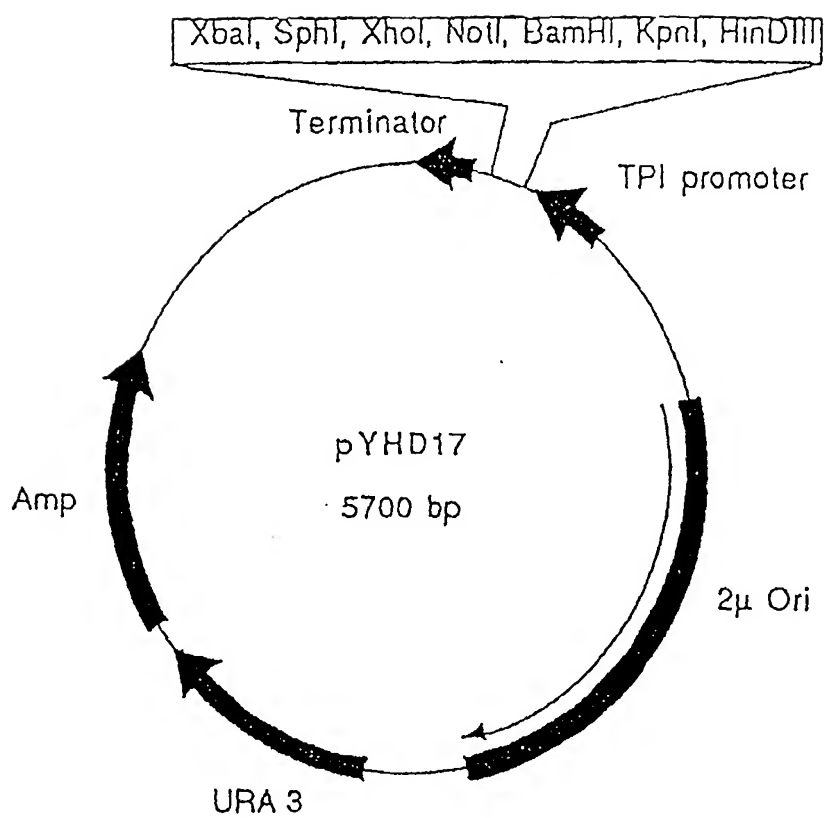


Fig. 1

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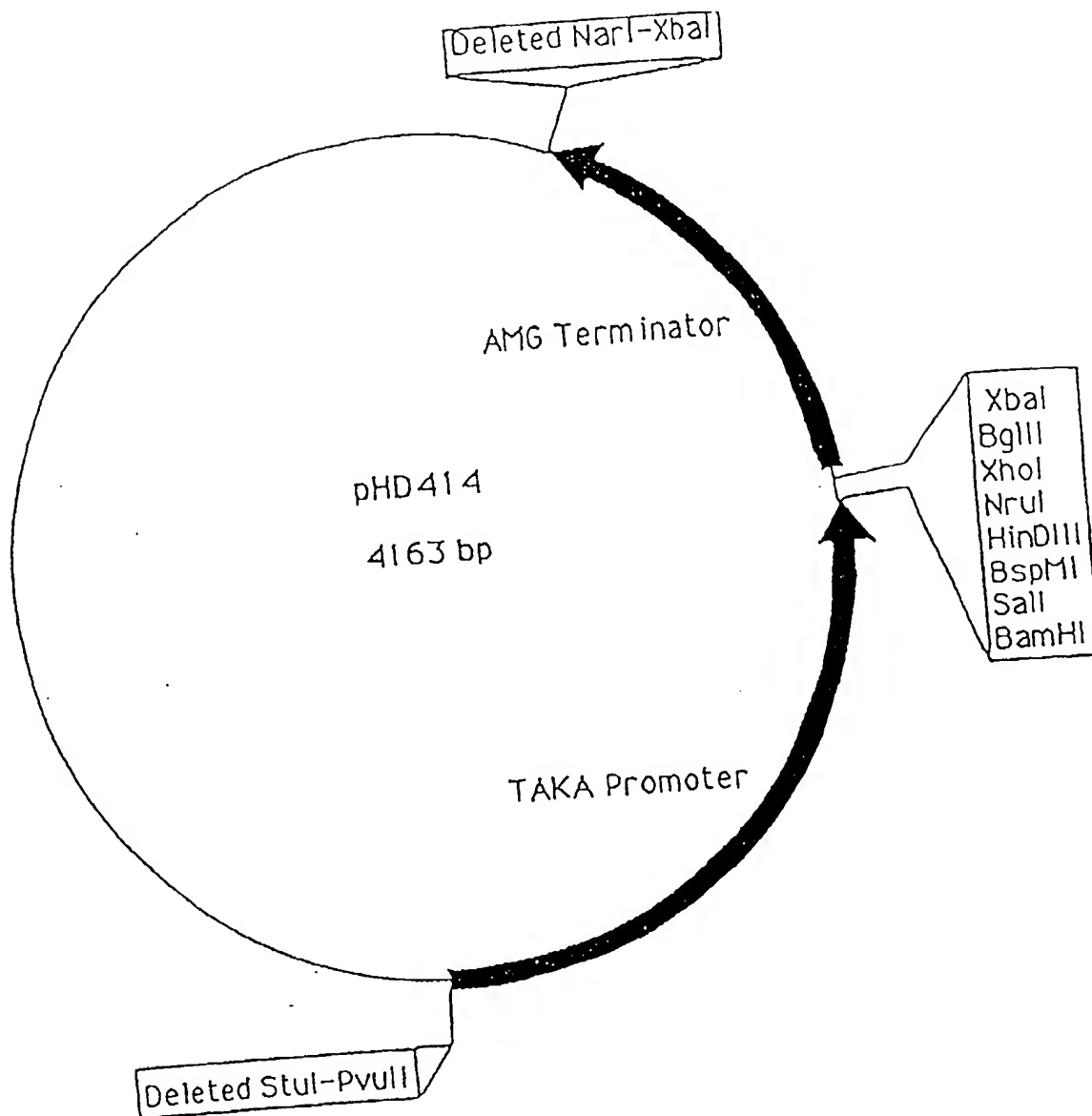


Fig. 2

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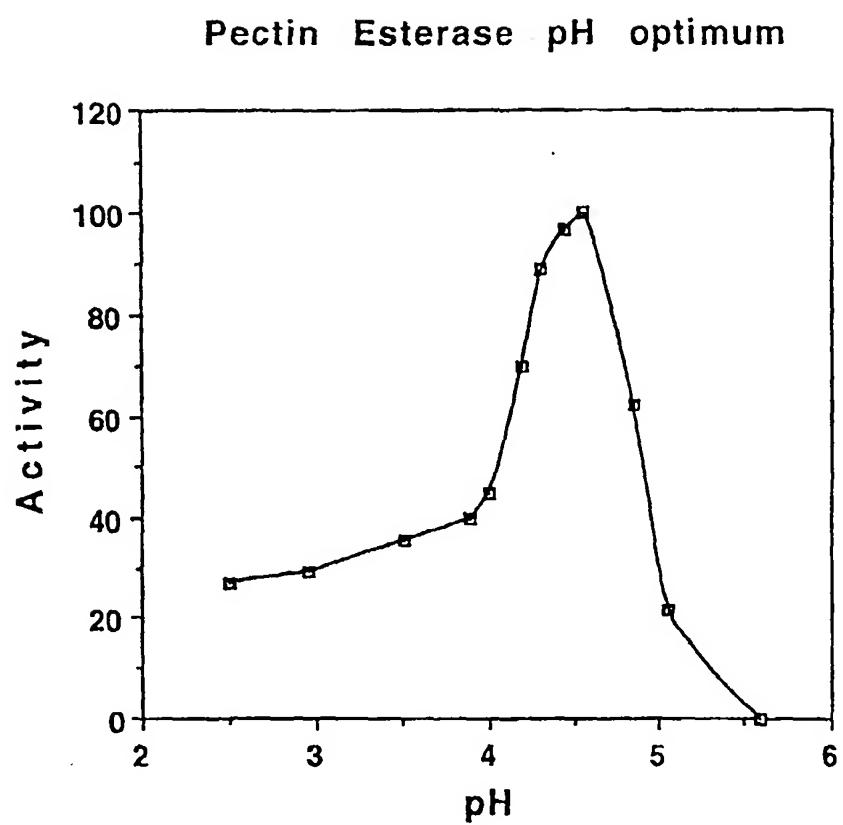


Fig. 3

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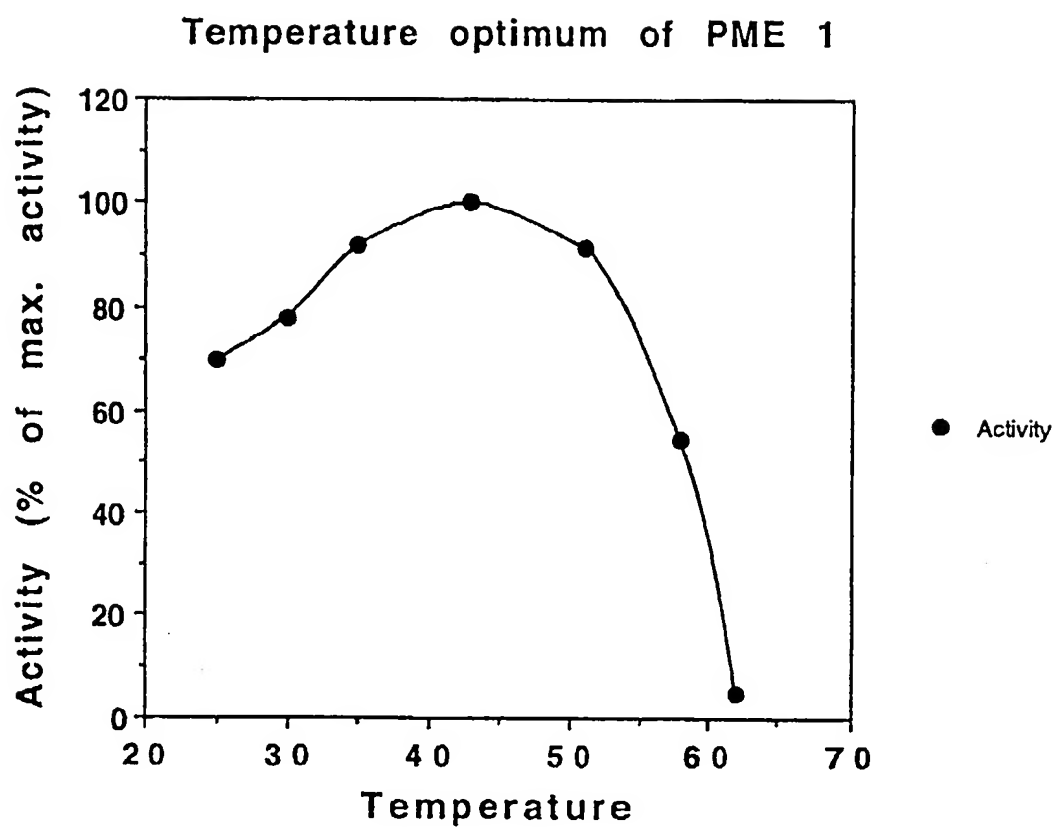


Fig. 4

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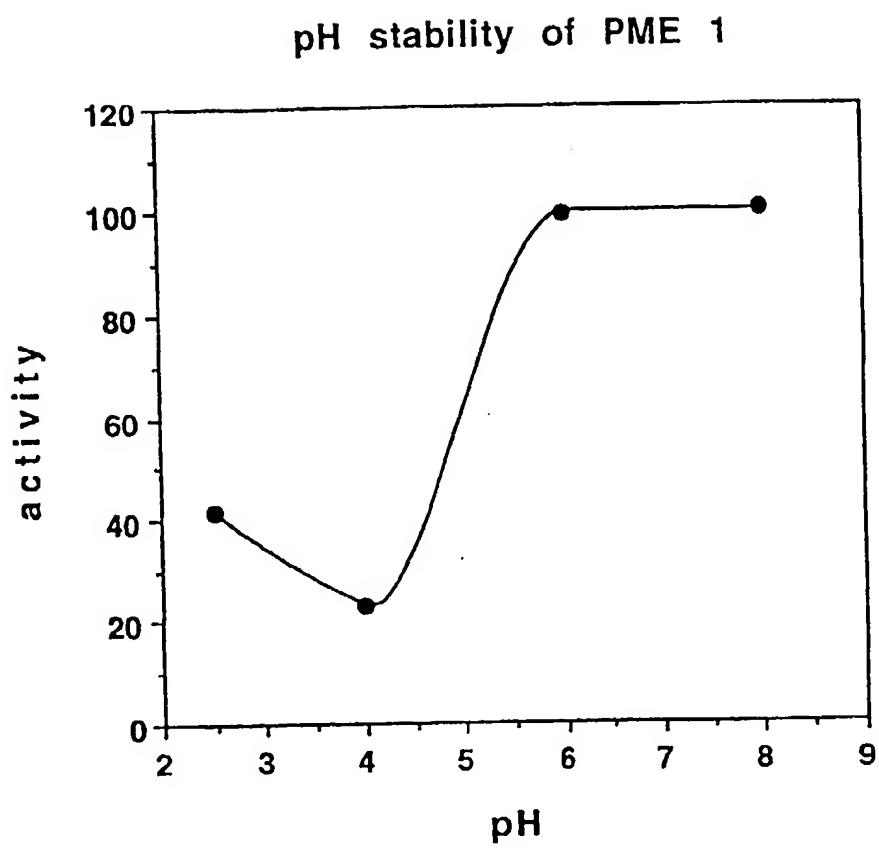


Fig. 5

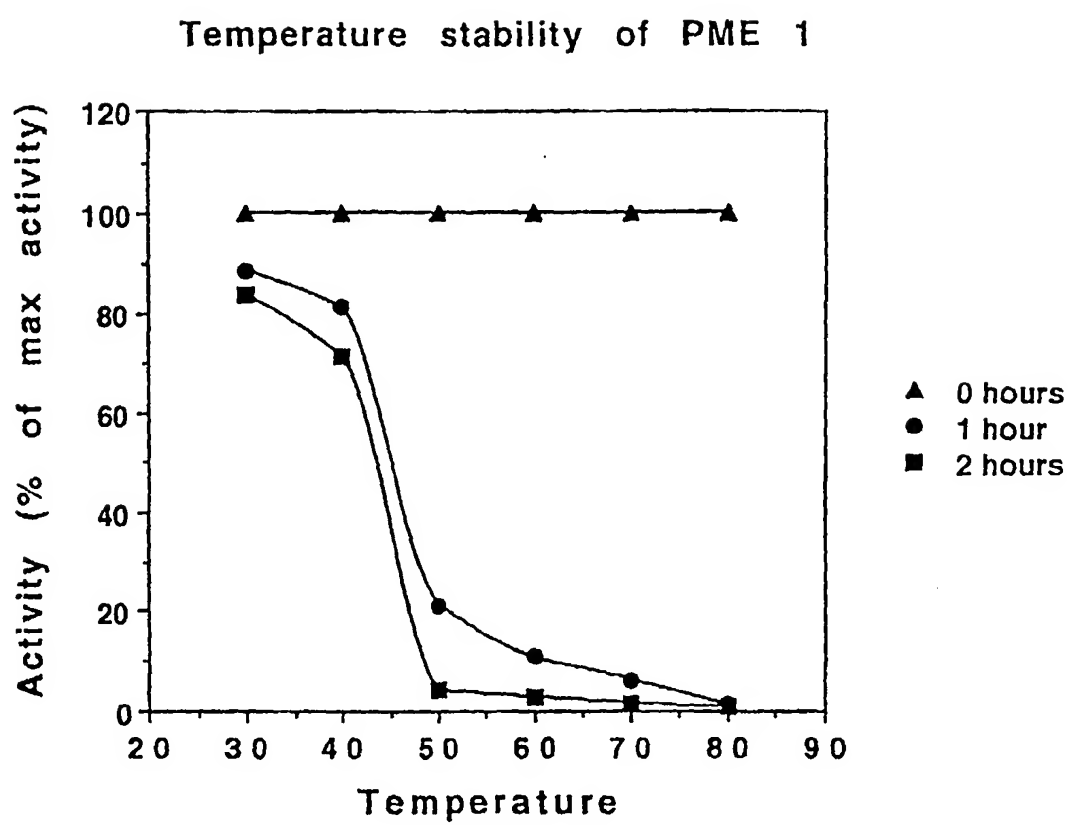


Fig. 6

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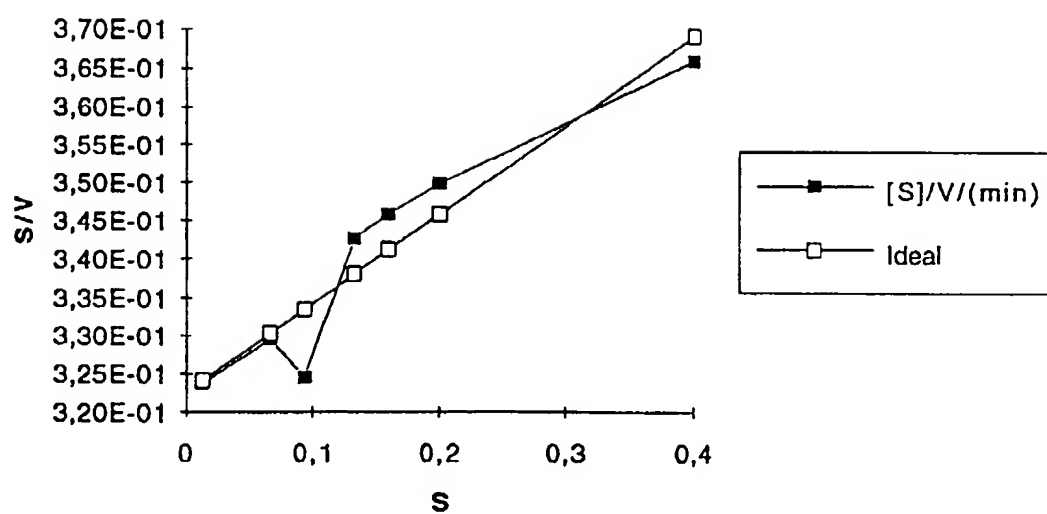


Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00173

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/18, C12N 15/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A1, 0388593 (RÖHM GMBH CHEMISCHE FABRIK), 26 Sept 1990 (26.09.90) --	1-21
X	Dialog Information Services, file 55, BIOSIS PREVIEWS, Dialog accession no. 9055724, Biosis no 93040724, Khanh N Q et al: "Characterization and expression of a genomic pectin methyl esterase- encoding gene in aspergillus-niger", & GENE (AMST) 106 (1). 1991. 71-78 --	1-21

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

15 Sept 1994

Date of mailing of the international search report

22 -09- 1994

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Palmcrantz
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00173

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 55, BIOSIS PREVIEWS, Dialog accession no. 7480061, Biosis no. 89131080, Markovic, O et al: "Purification and characterization of an aspergillus-niger pectin esterase", & BIOLOGIA (BRATISL) 44 (12). 1989. 1185-1190 --	1-21
X	US, A, 4200694 (SHIGETAKA ISHII ET AL), 29 April 1980 (29.04.80), the whole document, especially claim 1 -- -----	1-21

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00173

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1 (completely) and 4-21 (partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.